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14. ABSTRACT
Over the past decade, considerable progress has been made in understanding the molecular genetics of Tuberous Sclerosis (TSC), highlighted by the identification of the two tumor suppressor genes tsc1 and tsc2. Mutations in either tsc1 or tsc2 cause the disease TSC. A surge of recent research from several labs has shown that TSC1/2 antagonizes the mTOR (mammalian target of rapamycin) signaling network, which plays a central role in the regulation of cell growth in response to growth factors, cellular energy, and nutrient levels. In TSC1 or TSC2 mutant cells, the mTOR signaling pathway, as determined by the phosphorylation of S6K (ribosomal S6 kinase) and 4EBP1 (eukaryote initiation factor 4E binding protein), is highly elevated. Recent studies have also shown that TSC2 functions as a GTPase activating protein (GAP) to stimulate GTP hydrolysis of Rheb (a Ras family GTPase), therefore, inactivating Rheb. Both genetic and biochemical studies support that Rheb is a key direct downstream target of TSC2 and plays an essential role to mediate the physiological functions of TSC1/TSC2. The main objective of this project is to investigate the function of Herc in the regulation of TSC1/TSC2 stability and Rheb GTP level

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Introduction

This is the final report for the concept award W81XWH-05-1-0136. We proposed to investigate the function of Herc1 in the regulation of TSC2 and the Rheb GTPase. We have identified Herc1 as a TSC1 associated protein by immunoprecipitation and mass spectrometry analysis. Herc1 is a 532kDa protein with an E3 ubiquitin ligase HECT domain. We found that Herc1 promotes TSC2 ubiquitination and destabilizes TSC2. We were unable to detect any GEF activity of Herc1 towards Rheb. However, this negative data could be due to our inability to express the full length Herc1, which is a gigantic protein. We also showed that Herc1 stimulates S6K phosphorylation, possibly by decreasing TSC2 protein, which inhibits S6K phosphorylation. Our research provides a potential mechanism to modulate TSC2 tumor suppressor protein levels by interfering the TSC2 ubiquitination and degradation.

Body

Herc1 promotes TSC2 degradation and ubiquitination

Herc1 is an extremely large protein [1]. We have identified Herc1 as a TSC2 interacting protein. Interestingly, Herc1 contains an E3 ubiquitin ligase domain localized in the C-terminal region. We have found that the C-terminal domain of Herc1 interacted with TSC2 but not TSC1. TSC1 is known to interact with TSC2 [2]. Furthermore, the interaction between Herc1 and TSC2 is disrupted by the presence of TSC1 (Figure 1). These results suggest a model that TSC1 may stabilize TSC2 by disrupting the interaction between TSC2 and Herc1. We then tested this hypothesis by first examining whether Herc1 regulates TSC2 stability. TSC2 was co-transfected with the C-terminal region of Herc1 (residues 3901-C). The transfected cells were treated with cycloheximide to block new protein synthesis. The stability of TSC2 was measured. We found that co-expression of the C-terminal region of Herc1 significantly destabilized TSC2 in HEK293 cells (Figure 2). Furthermore, we examined the effect of Herc1 on TSC2 ubiquitination. We observed that TSC2 ubiquitination was dramatically decreased in cells treated the Herc1 RNAi, which should decrease Herc1 protein (Figure 3). Furthermore, we found that TSC1 indeed prevents the destabilizing effect of Herc1 on TSC2. Together, our data strongly indicate that Herc1 plays an important role in TSC2 ubiquitination and degradation.

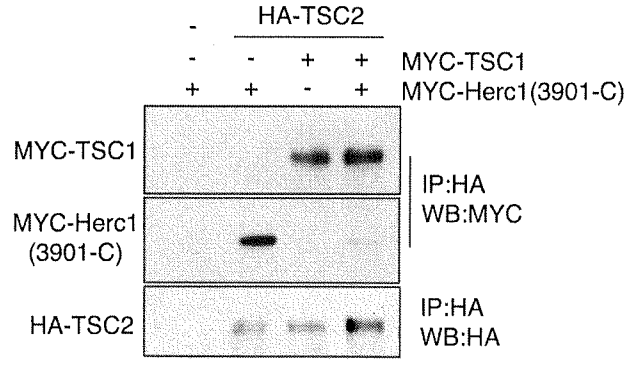

Figure 1. TSC1 disrupts the interaction between TSC2 and Herc1. HA-TSC2 was co-transfected with Myc-Herc1 in the presence or absence of Myc-TSC1 as indicated. Cells lysates were precipitated with anti-HA for HA-TSC2. The co-precipitated Myc-Herc1 or Myc-TSC1 were

detected by Western blot with anti-Myc. Co-expression of Myc-TSC1 dramatically decreased the Myc-Herc1 co-immunoprecipitated with HA-TSC2.

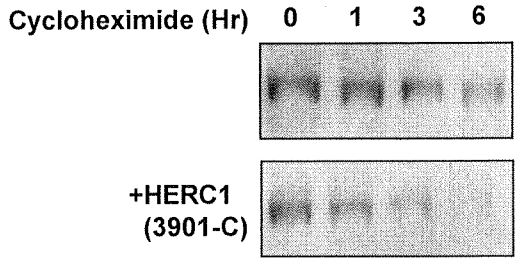

Figure 2. Expression of the C-terminal domain of Herc1 (3901-C) destabilizes TSC2. Myc-TSC2 was co-transfected with or without Herc1 into HEK293 cells. The transfected cells were treated with cycloheximide for indicated time (hours). The levels of Myc-TSC2 were determined by anti-Myc Western blotting and shown in the figure.

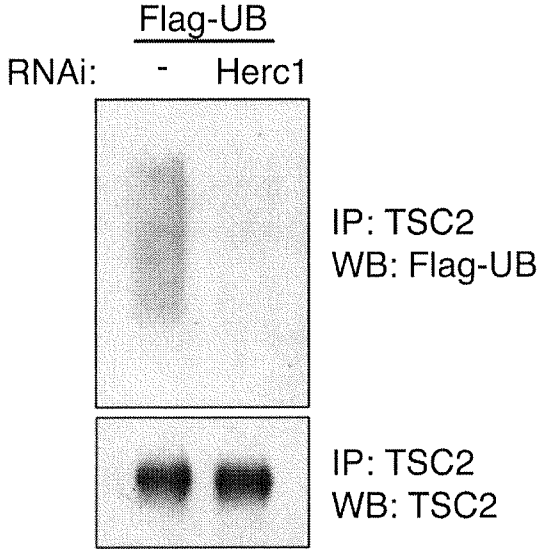

Figure 3. Herc1 regulates TSC2 ubiquitination. HEK293 cells were transfected with Flag-UB in the presence or absence of Herc1 RNAi as indicated. TSC2 was immunoprecipitated and ubiquitination was detected by anti-Flag Western blotting. Knockdown of Herc1 by RNAi decreased TSC2 ubiquitination.

Herc1 has no Rheb GEF activity

Herc1 contains two RLD domains, which have been implicated as GEF for the Ran family GTPase [3]. We performed experiments to test whether Herc1 may have GEF activity towards Rheb. We could not use S6K phosphorylation as an indirect assay for putative Herc1 GEF activity because Herc1 can stimulates S6K phosphorylation by decreasing TSC2 protein (see results in the previous section). Therefore, we expressed and purified the RLD domain (putative GEF for the Ran family GTPase) of Herc1 in E. coli as a GST-fusion. We measured Rheb guanine nucleotide exchange using purified recombinant proteins. We were unable to detect any GEF activity. Because Herc1 is a large protein it is difficult to be expressed in E. coli (or any other cells). Therefore, our data cannot exclude the possibility that the full-length protein of Herc1 is required for GEF activity.

Herc1 positively regulates S6K phosphorylation

Our data suggest that Herc1 may positively regulate mTOR activity by decreasing TSC2. To further support a role of Herc1 in mTOR regulation, we measured S6K phosphorylation in cells with Herc1 knockdown. HEK293 cells were transfected with interference RNAi for either TSC2 or Herc1. HA-S6K was co-transfected. Phosphorylation of S6K was determined. We found that knockdown of Herc1 decreased S6K phosphorylation (Figure 4). As predicted, knockdown of TSC2 increased S6K phosphorylation. These data demonstrate Herc1 as a positive regulator of the mTOR pathway and further support Herc1 as E3 ubiquitin ligase for TSC2.

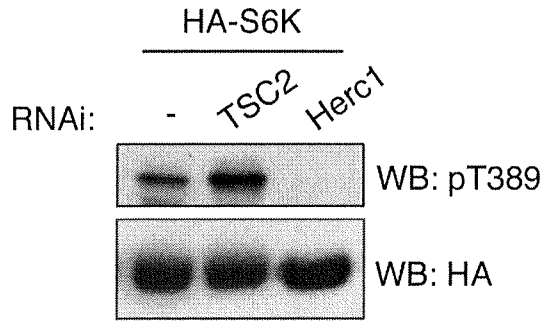

Figure 4. Herc1 and TSC2 have opposite effects on S6K phosphorylation. HA-S6K was co-transfected into cells in the presence of either TSC2 RNAi or Herc1 RNAi. HA-S6K was immunoprecipitated and phosphorylation of S6K was detected by anti-phospho-S6K antibody.

Key Research Accomplishments

1. Herc1 promotes TSC2 ubiquitination and degradation
2. The purified PLD domain of Herc1 displays no GEF activity towards Rheb in vitro
3. Herc1 stimulates S6K phosphorylation, likely by decreasing the TSC2 protein level

Reportable Outcomes

I have presented our findings at the National Cancer Institute (January 2006); the LAM Research Conference (March, 2006); American Association for Cancer Research (April 2006).

We have established TSC2 ubiquitination assay and constructed epitope tagged ubiquitin. We have provided plasmids to many research investigators in the scientific community.

Conclusions

Our findings suggest that Herc1 is an E3 ubiquitin ligase involved in ubiquitination and degradation of TSC2. We propose that TSC1 stabilizes TSC2 by interfering the association between TSC2 and Herc1. In the absence of TSC1, such as those TSC patients with mutations in TSC1, Herc1 destabilizes TSC2 and causes mTOR activation. Approximately 35% of TSC patients contain TSC1 mutation [4]. Therefore, our study indicates Herc1 as a potential therapeutic target for TSC patients caused by mutations in TSC1. In TSC1 mutant patients, mTOR is activated because Herc1 interacts with and destabilizes TSC2 in the absence of TSC1. We propose that inhibition of Herc1 should result in stabilization of TSC2, therefore, leading to inactivation of mTOR and inhibition of cell growth.

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Appendices

N/A